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Dealing with salinity extremes and nitrogen limitation – an unexpected strategy of the marine bacterium *Dinoroseobacter shibae*

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Summary

Having the right coping strategy for changes in osmolarity or desiccation is essential for the survival of every cell. So far, nothing is known about compatible solutes and the salt adaptation of the marine *Rhodobacteraceae*. The family member *Dinoroseobacter shibae* DFL12^T is shown here to form the compatible solutes α-glucosylglycerol (GG) and α-glucosylglycerate (GGA). To our knowledge, this is the first experimental evidence for GGA formation within the α-proteobacteria. Together with glutamate and putrescine, these substances enable good growth in salinity ranging from 0.3% to 5%. A salinity of 5% leads to a biomass share of 7.6% of compatible solutes and the very low salt level of 0.3% results in an 18-fold increased putrescine concentration compared with environmental conditions. Additionally, the substitution of glutamate by GGA has been shown during exposure to nitrogen limitation and in the stationary growth phase of the organism. Salt shock transcriptome analysis of *D. shibae* has revealed the essential role of its 153 kb chromid, which carries the genes for GG biosynthesis and several transport and exchange systems. Within the family of *Rhodobacteraceae*, the genomic capability of forming GG and GGA is strictly restricted to marine family members.

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Introduction

The marine environment comprises a number of special challenges for microorganisms. The high salinity, in particular, with regional and seasonal variations at the surface (Reul *et al.*, 2014), together with the mostly limited nutrient supply, which improves during times of, for example, collapsing algal blooms (Cloern, 1996; Rivkin and Anderson, 1997), have a major impact on survival.

In order to counteract salt stress, organisms have developed several strategies, one of which is the accumulation of organic compounds, the so-called compatible solutes. These are low-molecular-mass organic compounds that can be accumulated intracellularly in high concentrations without interfering with cellular metabolism (Brown, 1976).

During a change to hyperosmotic or hypoosmotic conditions, bacteria have to deal with a rapid water efflux or influx. The first response to these life-threating changes is osmoregulation with potassium ions. In a second step, cells accumulate compatible solutes by *de novo* synthesis or import them from their environment (Csonka, 1989). Compatible solutes are carbohydrates and their derivatives, polyoles and amino acids and their derivatives. Negatively charged molecules such as glutamate act mostly as a counter-ion for cations and are therefore also protective. Carbohydrate derivatives such as trehalose or glucosylglycerol probably act in a water exclusion mode, whereby a hydration shell of macromolecules is protected (Roeßler and Müller, 2001; Hagemann, 2011). In the marine environment, carbohydrate derivatives, such as galactosylglycerol in red algae and glucosylglycerol in cyanobacteria and marine pseudomonads, are common representatives (Karsten *et al.*, 1993; Mikkat *et al.*, 2000; Hagemann, 2011).

Recently, the Roseobacter group, a subgroup of the family *Rhodobacteraceae*, has been intensively investigated (Buchan *et al.*, 2005; Newton *et al.*, 2010; Hahnke *et al.*, 2013). In particular, two organisms of this group, namely *Dinoroseobacter shibae* DFL12^T (Biebl *et al.*, 2005) and *Phaeobacter inhibens* DSM 17395, have been studied with respect to their metabolism under various environmental challenges (Zech *et al.*, 2013a,b; Laass *et al.*, 2014; Wiegmann *et al.*, 2014). *D. shibae* is used as a model organism

for the marine *Rhodobacteraceae*, because of the various strategies that it uses to confront environmental challenges. Its capability for denitrification enables anaerobic growth (Piekarski *et al*., 2009; Laass *et al*., 2014); light can be used as additional energy source (Tomasch *et al*., 2011), which is an advantage in times of starvation (Soora *et al*., 2015) and the mutualistic life style with an algal host has been investigated (Wang *et al*., 2015).

Knowledge concerning osmoregulation in the marine Roseobacter group is so far very limited (Newton *et al*., 2010). In this article, we describe the identification and condition-specific production of newly identified compatible solutes in *D. shibae* grown under conditions of various medium salinities and nitrogen limitation.

Results

Identification of compatible solutes accumulated by D. shibae *DFL12*^{T}

Depending on the growth phase, four compounds dominate the metabolome of *D. shibae* DFL12^T as analysed by non-targeted GC-MS metabolome analysis. One of them is glutamate the others are substances with similar EI-MS spectra and no entries in established GC-MS databases.

For the identification of these unknown compounds, GC-APCI(+)-MS measurements were performed following the method of Strehmel *et al*. (2014) in parallel with GC-EI-MS. The assignment of the relevant compounds was achieved via a comparison of the retention times and the corresponding spectra. Although APCI is considered to be a soft ionization technique, strong in-source fragmentation of the investigated compounds was observed in this case. Equivalent phenomena have previously been reported for metabolites that consist of multiple silylated hydroxyl groups such as *O*-glucosides (Strehmel *et al*., 2014).

EI spectra of the unknown compounds showed fragmentation patterns typical for pyranoses (*m/z* 191, 204 and 217) plus a fragment with *m/z* 361 indicating a glycosidic linkage to another moiety (Simoneit *et al*., 2004). On the basis of this information, the protonated molecular ions were identified and the accurate masses $[M+H]^+$ of the substances and predicted sum formulae were determined from the GC-APCI(+)-MS spectra. The resulting mass ratios were: (i) *m/z* 685.3290, $C_{27}H_{65}O_8Si_6$ (mSigma: 8.8, |Δmass| 0.75 mDa, RI 2251), (ii) *m/z* 627.2687, $C_{24}H_{55}O_9Si_5$ (mSigma: 50.6, |Δmass| 0.90 mDa RI 2288) and (iii) *m/z* 699.3084 and $C_{27}H_{63}O_9Si_6$ (mSigma: 10.0, |Δmass| 0.22 mDa RI: 2309). When derivatization reagents and chemical ionization are taken into account, this leads to substances with the following sum formulae: $C_9H_{16}O_8$, $C_9H_{14}O_9$ and $C_9H_{14}O_9$, indicating that the two last-mentioned substances are presumably two derivatives (five and six trimethylsilyl moieties) of the same substance.

Based on the results from the GC-MS analyses, the genome of *D. shibae* was screened for potential enzymes involved in the biosynthesis of pyranoses glycosidically linked to a 3C-moiety. A gene annotated as glucosylglycerol-phosphate synthase (Dshi_3832, GgpS, EC 2.4.1.213) and a gene encoding a hypothetical protein with a BLASTp hit for glucosyl-3-phosphoglycerate synthase (Dshi_1821, GpgS, EC 2.4.1.266, E-value 4.5 e-117, annotation based on EnzymeDetector (Quester and Schomburg, 2011) were identified, being the only two genes encoding suitable enzymes. On the basis of these function predictions, the unidentified compounds were initially assumed to be the dephosphorylated enzyme products α-glucosylglycerol (GG, $C_9H_{18}O_8$) and α-glucosylglycerate (GGA, $C_9H_{16}O_9$), although a mass discrepancy of two protons was present for both metabolites. GG was supplied by bitop AG (Witten, Germany) and GGA was enzymatically synthesized in our laboratory by an *E. coli* expression clone (Klähn *et al*., 2010) and the method of Klähn *et al*. (2010). The analysis of these reference substances via GC-ACPI(+)-MS showed identical sum formulae (also with the loss of two protons (2.0085 mDa). The identical spectra and retention indices (also checked in spiking experiments, data not shown) confirmed the identification of GG and GGA (two derivatives) in the metabolome of *D. shibae*. Whether the two missing protons are a result of the chemical derivatization or ionization remains unclear.

The two substances were first identified in the cyanobacterium *Agmenellum quadruplicatum* (Kollman *et al*., 1979). In *Synechocystis* sp. PCC 6803, GG is synthesized in a two-step pathway starting from glycerol-3-phosphate and ADP-glucose via the intermediate glucosylglycerol phosphate (Hagemann and Erdmann, 1994). In *D. shibae*, the dephosphorylation of the intermediate is probably catalyzed by an enzyme encoded by Dshi_3831, currently annotated as sucrose-6F-phosphate phosphohydrolase, which is in the same operon as the synthase (Dshi_3832). In addition to the above-mentioned cyanobacteria and the heterotrophic genera *Stenotrophomonas* and *Pseudomonas* (Pocard *et al*., 1994; Mikkat *et al*., 2000; Roder *et al*., 2005; Hagemann *et al*., 2008; Hagemann, 2011), GG is formed by a wide range of organisms including the plant *Myrothamnus flabellifolia* and probably by the fungus *Aspergillus oryzae* (Bianchi *et al*., 1993; Takenaka and Uchiyama, 2000).

The synthesis of GGA is a two-step pathway equivalent to GG (Costa *et al*., 2006, 2007) starting from 3-phosphoglycerate instead of glycerol-3-phosphate. The dephosphorylation step is probably catalyzed by the enzyme encoded by Dshi_1820, currently annotated as mannosyl-3-phosphoglycerate phosphatase in the same

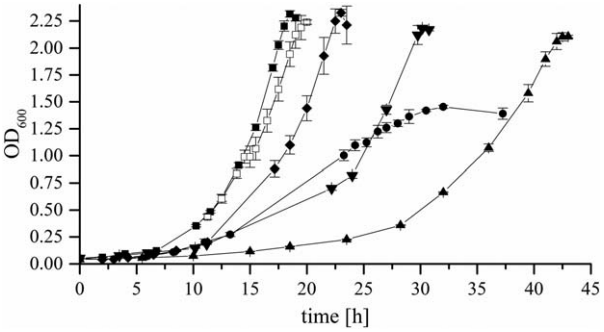

Fig. 1. Growth behavior of *D. shibae* DFL12^T at various salinities, after osmotic shock or under nitrogen limitation. ▲: 0.3% salinity, ■: 2.3% salinity, ◆: 3.5% salinity, ▼: 5% salinity, ●:3.5% salinity and nitrogen-limited, □: initial salinity 2.3% with shift to 5% salinity at an OD of 1. Salinity is represented by 0.3% basic salt mix and a corresponding amount NaCl (w/v). OD, optical density.

operon as the synthase (Dshi_1821). Until now, no experimental evidence for the occurrence of GGA in α-proteobacteria was found. It has been found in cyano- and γ-proteobacteria and in the archaeon *Methanohalophilus portucalensis*, the aquificae *Persephonella marina* and, recently, in the plant *Selaginella moellendorffii* (Robertson *et al.*, 1992; Cánovas *et al.*, 1999; Goude *et al.*, 2004; Costa *et al.*, 2007; Empadinhas and da Costa, 2008b; Nobre *et al.*, 2012).

Growth behaviour of D. shibae *DFL12^T exposed to various salinities and nitrogen limitation*

We observed growth of *D. shibae* at various salt concentrations ranging from almost fresh water conditions of 0.3% salinity [basic salt mix (experimental procedures) expressed as NaCl (w/v)] up to 5% [4.7% NaCl plus basic salt mix (w/v), Fig. 1]. The fastest growth occurred in medium containing 2.3% salt [2% NaCl plus basic salt mix (w/v)], about 65% of the sea water concentration. With rising salt concentrations, the growth rate decreased to a level at which the growth of cells was strongly restricted (7% salinity, data not shown). A very low salinity also led to strongly impaired growth, as expected for a moderately halotolerant organism.

Cells grown at an initial salinity of 2.3% discontinued growth for about 30 min when experiencing a salt shock at 5%. Growth subsequently recommenced, with growth behaviour being similar to that of untreated cells.

Cells grown in a nitrogen-limited medium (1 mM ammonium instead of 4.7 mM) and a salt concentration of 3.5% (3.2% NaCl plus basic salt mix w/v) showed strongly hampered growth. The growth behaviour changed and the maximal optical density decreased compared with *D. shibae* grown at 3.5% salinity (Fig. 1).

Influence of various salinities on production of glutamate, glucosylglycerol and glucosylglycerate

The production of glutamate, α-glucosylglycerol and α-glucosylglycerate of long-term-adapted *D. shibae* cells was investigated via intracellular metabolome analysis in the mid-exponential phase and at maximal optical density (OD_{max}) when grown in SWM with various salinities (0.3%, 2.3%, 3.5% and 5% (w/v).

Under all conditions tested, glutamate was the most abundant compatible solute in *D. shibae* cells, independent of growth phase and salinity (with respect to GC-MS peak area). Although it showed only small differences in the exponential phase under 2.3%, 3.5% and 5% salinity, the glutamate concentration was more than fivefold decreased in cultures grown under 0.3% salinity (Fig. 2). Compared with its concentration in the exponential phase at OD_{max}, glutamate was strongly reduced under all conditions, except in cells grown in medium containing 5% salt in

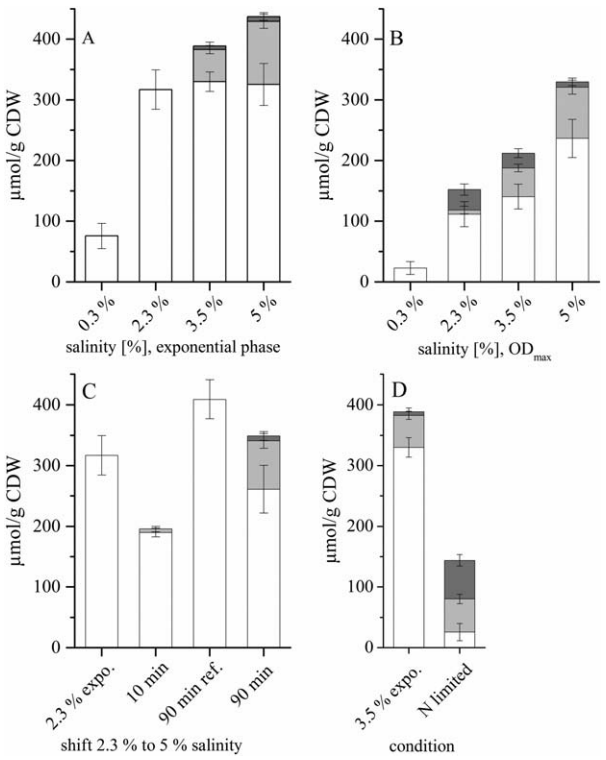

Fig. 2. Distribution of the compatible solutes glutamate (open bars), α-glucosylglycerol (light grey bars) and α-glucosylglycerate (dark grey bars) in dependence on growth phase, salinity and nitrogen supply and in osmotically shocked cells. CDW, cell dry weight. Error bars, standard deviation. Salinity influence in A. exponential growth and B. at OD_{max}. C. Changes in compatible solute distribution at 10 and 90 min after salt shock compared with untreated reference cultures at the same time points (2.3% expo. and 90 min ref.). D. Changes in compatible solutes distribution under nitrogen limitation compared with reference condition (3.5% salinity, exponential). Salinity represented by 0.3% basic salt mix and a corresponding amount NaCl (w/v).

which the effect was weak (Fig. 2). At 0.3% salinity, glutamate was the only compatible solute detected.

α-Glucosylglycerol was first detected in cells grown under marine environmental conditions (3.5% salts) during the exponential phase. Its concentration increased with salinity and growth (Fig. 2).

In contrast, α-glucosylglycerate seemed to be less dependent on salinity, compared with GG, but was strongly dependent on the growth phase. It was first detected in the transition state prior to the stationary phase (2.3% salinity) and in low but consistent amounts throughout cultivation in medium with 3.5% or 5% salinity (Fig. 2).

Response of compatible solute concentrations to salt shock and nitrogen limitation

D. shibae cells in the exponential phase were transferred from a low salt level (2.3%) to a high level (5%) and were then analyzed via GC-MS. GG, which was not present in the cells before transfer, was detected just 10 min after the shock, whereas the glutamate content decreased by about 30%. The concentration of GG increased constantly during the following 80 min, whereas GG was not detectable in the parallel reference cultures grown at a salt concentration of 2.3% (Fig. 2). During progressive growth, glutamate retained its low level and little GGA was detected.

Cells grown under nitrogen limitation (salinity 3.5%, ammonium: 1 mM) contained a significantly larger amount (259-fold change) of GGA compared with all other conditions tested. In these cells, GGA represented the most abundant substance under these conditions. Whereas the GG concentration was comparable to the reference condition (3.5 salinity, exponential phase), the concentration of glutamate decreased more than 15-fold under nitrogen limitation (Fig. 2).

Metabolome analysis

The concentration of 55 identified metabolites was monitored under the described conditions. A complete list of the detected metabolites and their fold changes (reference state: exponential phase and OD_{max} at 3.5% salinity) throughout the experiments is available in the supporting information (Supporting Information Table S1). A hierarchical cluster analysis resulted in four clusters (Fig. 3). Cluster I comprises the exponential and OD_{max} at 0.3% salt. Cluster II comprises the three conditions: OD_{max} of cultures at 2.3% and 3.5% salinity and nitrogen limitation. Cluster III consists of the three conditions: 5% salt (exponential and OD_{max}) and 90 min after salt shock. Finally, cluster IV includes the four conditions: exponential phase of cultures at 2.3% and 3.5% salinity, 10 min after salt shock and the 90 min reference culture (Fig. 3). Overall,

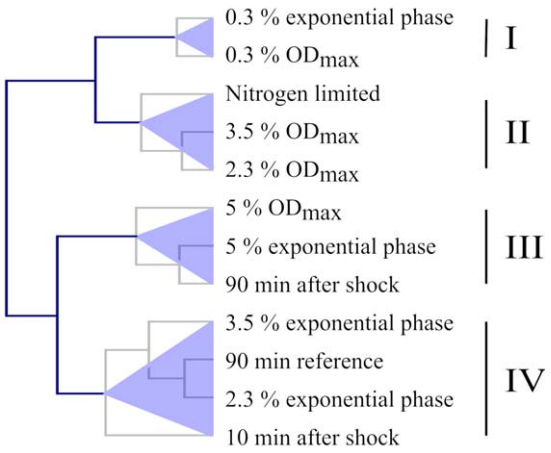

Fig. 3. Cluster analysis based on metabolite concentrations using Pearson correlation (TIGRMeV4 (Saeed *et al.*, 2003). [Colour figure can be viewed at wileyonlinelibrary.com]

only slight metabolic differences are seen between the moderate salt concentrations of 2.3% and 3.5%. The more extreme conditions of 0.3% and 5% salinity are clearly separated and thus indicate that distinct adaptation processes are needed.

D. shibae cells grown at 0.3% salinity showed a very low content of amino acids under exponential growth (fold change 0.89–0.04); this was less distinct at OD_{max}, compared with the reference conditions (Supporting Information Table S1). Otherwise, remarkable amounts of putrescine (fold change 17.91) and the two lipid metabolism intermediates glycerol-3-phosphate (fold change 8.07) and 11-octadecenoate (fold change 3.53) were detected.

Nitrogen-limited cells showed a strongly reduced pool of nitrogen-containing substances, with the exception of tyrosine, which was found to be increased (Supporting Information Table S1).

Further observed metabolic changes were unremarkable in the context of salinity and are therefore only discussed to a limited extent in the following.

Cell response to salt shock at the transcriptional level

To understand the adaptation process of *D. shibae* during salt shock, a whole genome RNA-Seq analysis was performed. In this analysis, 3873 gene transcripts (coverage of 91.3%) were detected of which 1159 genes encoded hypothetical proteins (Wagner-Döbler *et al.*, 2010). At 10 min and 90 min after salt shock, 382 and 113 transcripts were regulated with a $\log_2$ ratio greater or less than ± 2 respectively (Supporting Information Table S2).

The upregulation of 42% of the genes of the 153 kb chromid at 10 min after the salt shock (Table 1) was conspicuous. Even 90 min after the shock, approximately 23%

Table 1. Number of upregulated and downregulated genes (cutoff: $\log_2$ ratio of ±2) 10 and 90 min after the salt shock.

	upregulated		downregulated		% regulated	
Location	10 min	90 min	10 min	90 min	10 min	90 min
Chromosome	172	21	100	73	8.1	2.8
Plasmid 191 kb	18	1	3	1	10.8	1.1
Chromid 153 kb	57	13	–	–	41.9	9.6
Plasmid 126 kb	12	–	–	–	17.1	–
Plasmid 86 kb	2	–	3	–	8.5	–
Chromid 72 kb	15	4	1	–	26.2	6.6

of these genes were still upregulated. This indicates the essential role of the 153 kb chromid in the adaptation to higher salinity; the chromid carries the genes encoding GG biosynthesis, numerous transporters and channels and some hypothetical proteins. In contrast, the regulation of the genes of the chromosome and the other extrachromosomal elements decreased again or inverted during the adaptation process.

The short term response to salt shock includes the transcriptional upregulation of genes encoding general stress proteins (Table 2), uncharacterized transcriptional regulators and Dshi_3834, a signal transduction histidine kinase ($\log_2$ fold-change 5.6), exchange systems such as transporters or channels (Table 2), genes encoding enzymes for glucosylglycerol biosynthesis and the Entner–Doudoroff pathway (Table 2), plus several tRNAs' and ribosomal components such as 23S ribosomal RNA (Supporting Information Table S2).

The transcript levels of the genes that probably encode the enzymes for GGA formation were essentially the same after salt shock. Only Dshi_1820 encoding the phosphatase was upregulated with a $\log_2$-ratio of 1.7 90 min after salt shock but was unregulated directly after the applied shock.

By 90 min after the salt shock, most transcriptional levels had returned to the pre-shock state or were inverted. Genes for GG biosynthesis and some transporters and channels were still upregulated. These genes are mainly located on the 153 and 72 kb chromids.

Comparative genomics study

We investigated the resistance strategy against salt stress of the *Rhodobacteraceae* family in general and compared it with that of *D. shibae*. In particular, we examined whether the formation of GG and GGA could be assigned to habitat: in this case, marine versus non-marine.

The obtained phylogenetic tree shows the general taxonomy of selected *Rhodobacteraceae* (Fig. 4; Munoz *et al*., 2011; Pujalte *et al*., 2014). The genus *Oceanicola* is noticeable because of its distribution within the tree and has previously been proposed for reclassification several times (Newton *et al*., 2010; Gifford *et al*., 2014; Luo and Moran, 2014; Luo *et al*., 2014).

With BLAST analyses, the sequence-based distinction between trehalose-6-phosphatase (EC 2.4.1.15) and glucosylglycerolphosphate synthase (2.4.1.213) proved to be difficult on the whole family level. However, a close look at the protein domain architecture of the gene in question plus its neighbours allowed this distinction (Fig. 5).

EC 2.4.1.213 function has been established for the *Rhodobacteraceae Rhodovulum sulfidophilum* DSM1374 (Severin *et al*., 1992). A BLAST sequence comparison of *D. shibae* Dshi_3832 and *R. sulfidophilum* TY12_RS01660 resulted in an E-value of 0.0, an identity of 70% and a coverage of 98%. Hence, the protein domain architecture is the same for the two investigated organisms. The operon architecture mentioned above and therefore probably biosynthesis of GG is unique to the marine *Rhodobacteraceae* tested (Fig. 4). With regard to the genomes of the non-marine *Rhodobacteraceae* tested, only a predicted trehalosephosphate-UDP-glucosyltransferase (EC 2.4.1.15) function was found in a different genome context (Fig. 4). In all tested organisms either EC 2.4.1.213 in the mentioned genome context or EC 2.4.1.15 was found, but never both on the same genome.

As for GG biosynthesis, GGA synthesis genes were only determined in marine *Rhodobacteraceae*. Based on sequence similarities, a distribution of the corresponding operon was detected throughout the tree (Fig. 4). Marine *Rhodobacteraceae*, which consistently lacked both synthesis pathways, are members of the genera *Labrenzia*, *Leisingera*, *Phaeobacter* and *Roseovarius*. This indicates that the biosynthesis of GG and GGA are not essential for the marine lifestyle.

Overall, 41 organisms of the 135 organisms tested seem to be capable of GGA formation, 16 of GG formation and within these, nine organisms are capable of both.

Homologous genes to those involved in GG and GGA biosynthesis are found in marine and non-marine pseudomonades, in cyanobacteria, partly in plants, and in the case of GGA, also in the Archeae.

Nitrogen sources used by D. shibae *DFL12^T*

Phenotype MicroArray analyses showed a wide usage of inorganic nitrogen sources by *D. shibae*, which can however also employ organically fixed nitrogen. Out of the 95 tested nitrogen sources, 18 were positive. Respiration occurred with the inorganic substances ammonia, nitrate and urea, with the amino acids L- and D-alanine, L-aspartatic acid, L-glutamic acid and L-glutamine, with the nucleobases adenine, cytosine, guanosine and uracil and their degradation products allantoin, uric acid and xanthine and with glucuronamide, inosine and parabanic acid.

Table 2. Selected gene transcripts of D. shibae with corresponding log2 fold-changes 10 and 90 min after the salt shock. Ratios were computed using an untreated culture.

			log_2 fold-change	
Locus tag	Gene name	Product	10 min	90 min
Genes encoding general stress proteins				
Dshi_0107		Ferritin-like protein	0.9	2.7
Dshi_0169		Putative glutathione S-transferase	4.0	0.0
Dshi_0617	*clpB*	Chaperone protein clpB	1.8	2.8
Dshi_2892		Small heat shock (HSP20) protein	1.8	4.9
Dshi_3839		Ferroxidase	6.1	1.2
Dshi_3912		Ferroxidase	6.1	0.9
Dshi_4184		CsbD family protein	9.8	2.9
Genes encoding enzymes involved in glucosylglycerol biosynthesis				
Dshi_0630	*glpD*	Glycerol-3-phosphate dehydrogenase	6.0	5.4
Dshi_1742	*gap1*	Glyceraldehyde-3-phosphate dehydrogenase	5.4	−2.0
Dshi_3830		Glycerol-3-phosphate dehydrogenase	4.8	3.6
Dshi_3831		Glucosylglycerol-3-phosphatase[a]	4.7	4.0
Dshi_3832		Glucosylglycerol-phosphate synthase	5.1	3.4
Genes encoding enzymes of the Entner-Doudoroff pathway				
Dshi_1682	*pgi*	Glucose-6-phosphate isomerase	5.5	−0.6
Dshi_1683		Putative 6-phosphogluconolactonase	5.7	−1.5
Dshi_1684	*zwf*	Glucose-6-phosphate 1-dehydrogenase	3.1	−0.7
Dshi_1768	*eda*	KHG/KDPG aldolase	3.6	0.2
Dshi_1769	*edd*	Phosphogluconate dehydratase	3.9	−0.3
Genes encoding transporters or channels[b]				
Dshi_0144	*amtB*	Ammonium transporter	d	–
Dshi_0743	*mscS*	MscS mechano sensitive ion channel protein	d	–
Dshi_3001	*dctM2*	TRAP dicarboxylate transporter subunit DctM	3.3	0.2
Dshi_3036		Efflux transporter	3.6	−0.2
Dshi_3884		Multi-anti-extrusion protein MatE	d	–
Dshi_3897		MgtC/SapB transporter	d	d
Dshi_3902		RND family efflux transporter MFP subunit	d	–
Dshi_3905		MscS mechano sensitive ion channel	5.0	0.6
Dshi_3906		SSS family solute/sodium (Na+) symporter	7.1	−0.2
Dshi_3907		Sodium/calcium exchanger membrane region	5.1	1.1
Dshi_3913		TrkA domain-containing protein	3.6	0.6
Dshi_3914		Cation transporter	d	1.6
Dshi_3922		Transport protein, putative	5.7	1.7
Dshi_4182		MscS mechano sensitive ion channel	d	–

a. Primary annotation: sucrose-6F-phosphate phosphohydrolase.
b. Only transcripts with a fold change $\geq$3 are shown or "d" transcripts only reliably detected under shock conditions (threshold: expression value 4).

Discussion

D. shibae *DFL12^T can grow in a wide range of salinities*

In SWM, we could show that *D. shibae* DFL12^T grows in a wide salinity range between 0.3% and 5% [0.3% corresponds to basic salt mix (experimental procedures) expressed as NaCl and 5% consists of basic salt mix and 4.7% NaCl (w/v; Fig. 1)] but is strongly hampered at 7% salt (data not shown). In the literature, a minimal requirement of 1% salinity and good growth up to a salinity of 7% has been reported for the growth of *D. shibae* in a complex medium (Biebl *et al.*, 2005). The latter complex medium contains yeast extract, which is known to include the component glycine betaine, a strong osmolyte (Dulaney *et al.*, 1968; Empadinhas and da Costa, 2008a), which can probably be imported by *D. shibae* as shown for other organisms (Csonka, 1989); this might be an explanation for the increased salt tolerance reported. Hence, the finding that *D. shibae* is able to grow almost under freshwater conditions was unexpected. A similar result has been published for other moderately halotolerant organisms (Hagemann, 2011).

A striking result of the analysis is the comparably moderate effect of salt shock on growth (Fig. 1). The short interruption of growth is probably a result of in-streaming sodium ions, which are known to affect the metabolism strongly (Hagemann, 2011). After salt shock, sodium ions are actively replaced by the less harmful potassium ions, as has often been shown for other bacteria (Reed *et al.*, 1985; Roeßler and Müller, 2001). This is probably the

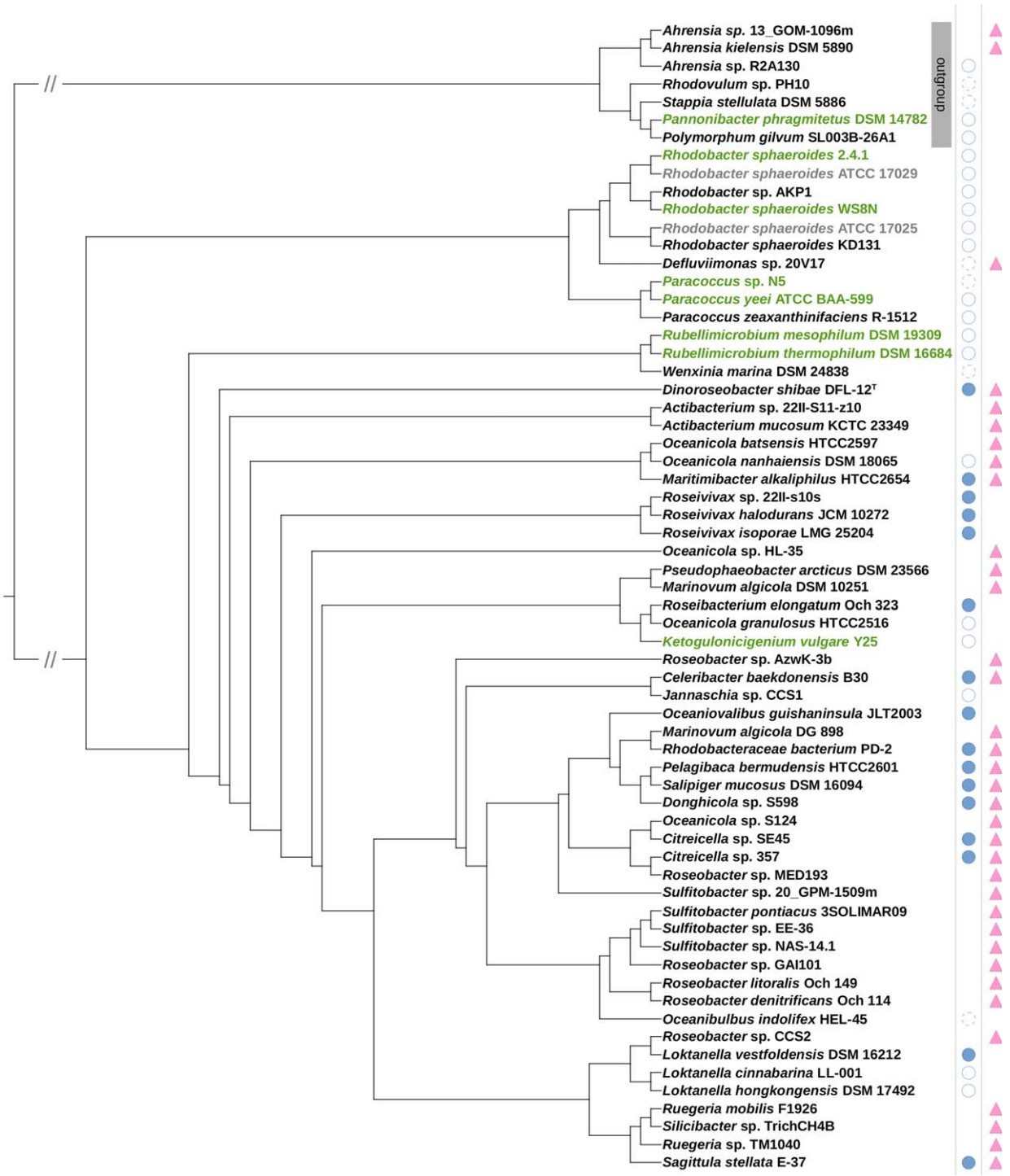

Fig. 4. Consensus 16S rRNA distance tree combined with maximum likelihood tree of 64 *Rhodobacteraceae* organisms for marine (black), non-marine (green) and unknown (grey) habitats rooted with seven sister family members as outgroup. Glucosylglycerol synthesis pathway is marked as completely detected if either HMM or BLAST hit candidate for *D. shibae* operon Dshi_3830 and for Dshi_3831, Dshi_3832 (EC 2.4.1.213) were found with an *E*-value better than 10^{-60} and an identity higher than 50% while the operon protein architecture possesses the same domains as the query proteins (●). Organisms having only a hit candidate for Dshi_3832 (EC 2.4.1.213) with an E-value better than 10^{-3} and an identity higher than 30% but with a different operon protein architecture containing a trehalosephosphatase domain were marked as mainly EC 2.4.1.15-type (○). Hits with several protein domain architectures or non assignable gene order in case of draft genomes are shown with dashed open circles (○).\r\nRhodobacteraceae possessing genes with better similarity than an *E*-value of 10^{-60} and an identity higher than 50% with respect to *D. shibae* Dshi_1820 and Dshi_1821 were tagged as being capable of glucosylglycerate synthesis (▲).

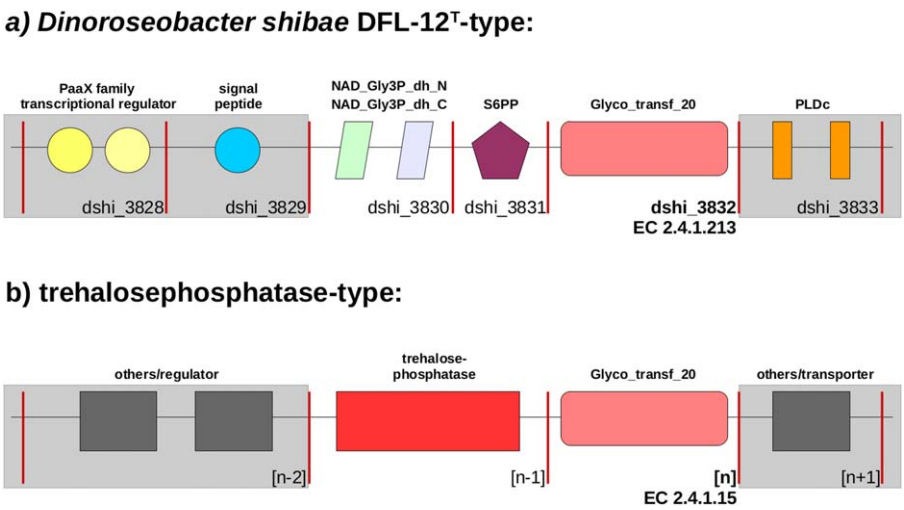

Fig. 5. Two different main types of glucosylglycerol synthesis operon protein domain architecture: consists of a glycosyl transferase family domain (Dshi_3832, E-value with respect to SMART domain 10^{-139}) coding for the enzymatic function EC 2.4.1.213 preceded by sucrose-6F-phosphate phosphohydrolase (Dshi_3831, *E*-value 10^{-38}) and two glycerol-3-phosphate dehydrogenases (EC 1.1.1.8) encoded on Dshi_3830 (*E*-value 10^{-44}). Signal peptides and transcriptional regulators are encoded upstream (Dshi_3828, Dshi_3829). The operon ends with two phospholipase D domains (Dshi_3833, *E*-value 10^{-44}) and other signal transduction or regulation factors (Dshi_3834/35). Phospholipase D (EC 3.1.4.4) may be essential for the formation of certain types of transport vesicles or constitutive for vesicular transport in signal transduction pathways (Finn *et al*., 2014).\nb) is mainly determined by the occurrence of a trehalosephosphatase domain preceding the glycosyl transferase on the operon. These candidates are assigned to the enzymatic function of a trehalosephosphate-UDP glucosyltransferase (EC 2.4.1.15) involved in the trehalose biosynthesis pathway (Chang *et al*., 2014).

same for *D. shibae*. After its successful rearrangement of ions, replication is possible once again, although proteomic adaptation has not yet finished and the GG concentration has not yet reached its final level at this time point.

Different and special compatible solutes in dependence of salinity and nitrogen supply

Using conditions comparable to brackish water [0.3%–2.3% salinity; 2.3% consists of 0.3% basic salt mix plus 2% NaCl (w/v)], the data presented here indicate that different counter-ions for cations are used to maintain the cellular turgor pressure of *D. shibae*. In particular, glutamate is used as the first choice counter-ion, providing that a surplus of nutrients is available. However, the nitrogen-limitation experiment indicates that GGA is an essential and adequate substituent for the nitrogen-containing glutamate under these conditions. A similar nitrogen dependence has been shown for the soil bacterium *Erwinia chrysanthemi* and for several cyanobacteria (Kollman *et al*., 1979; Goude *et al*., 2004; Klähn *et al*., 2010b), whereas other organisms are not known to use GGA as a compatible solute at all (Robertson *et al*., 1992; Cánovas *et al*., 1999). By using GGA, *D. shibae* maintains cellular turgor pressure, which guarantees not only survival, but also growth, although to a lesser extent (Fig. 1). The marine environment is a nitrogen-poor environment for non-N_2-fixing bacteria (Vitousek and Howarth, 1991; Rivkin and Anderson, 1997). As shown in the Phenotype MicroArray experiment, *D. shibae* can utilize amino acids and nucleobases as a nitrogen source. To a certain extent, the algae-associated *D. shibae* certainly relies on such organic nitrogen sources that are released during a collapse of algal blooms (Baines and Pace, 1991) or on direct exchange during its symbiosis with algae (Wagner-Döbler *et al*., 2010; Wang *et al*., 2014). Nevertheless, a nitrogen-independent strategy for osmoregulation is a strong advantage for survival. Without the capability of forming GGA, *D. shibae* would probably not be able to replicate or even to survive in its natural saline environment with nitrogen limitation. This shows the complexity of the interplay of several parameters, such as environmental factors, nutrients and stress tolerance. Only a few studies have investigated such interactions; for instance, a reduced temperature sensitivity of the marine bacterium *Halomonas hydrothermalis* has been shown during iron starvation (Harrison *et al*., 2015) and a certain minimum salinity promotes growth rate under low temperatures and high hydrostatic pressures in further *Halomonas* strains (Kaye and Baross, 2004).

When a certain threshold value of salt is reached, additional compatible solutes other than glutamate are needed. Hence, GG is formed by *D. shibae*. The accessory osmolyte GG leads to an improved salt tolerance as previously shown, for example, for *Synechocystis* sp. PCC 6803 and its Δ*ggpS* mutant. The loss of *ggpS* leads to reduced salt tolerance of around 40% (Ferjani *et al*., 2003). Even the salt tolerance of *Arabidopsis thaliana* can be improved by

transferring a *ggpPS* gene into its genome (Klähn *et al*., 2009). Moreover, two organisms of the bacterial genus *Stenotrophomonas* show different salt tolerance depending on their ability to form GG (Roder *et al*., 2005).

Overall, our data allow us to estimate the share of compatible solutes of the cell dry weight: under low salt conditions, the sum of glutamate, GG and GGA is 1.1 and, under high salt, the amount is 7.6% of the cell dry weight. For the non-marine *E. coli*, the percentage for the total soluble pool was reported to be 2.9% of cell dry weight (Neidhardt and Umbarger, 1996).

Metabolic response to salt shock

GG is formed just 10 min after salt shock (Fig. 3) and, concomitantly, transcripts for glucosylglycerol-phosphate synthase (GgpS) and glucosylglycerol 3-phosphatase (GgpP) are strongly upregulated at 10 min and even up to 90 min after salt shock (Table 2). However, an exclusive production of GG by induced enzymes, 10 min after salt shock, is excluded. Laass *et al*. have investigated the transcriptome and proteome of *D. shibae* during the transition from oxygen to nitrate respiration. In this study, *D. shibae* was shifted to anaerobic conditions, with the first essential enzymes for denitrification being detected after at least 30 min (Laass *et al*., 2014). An immediate GG formation attributable to osmotic shock has also been reported before for *Synechocystis* sp. PCC 6803 (Hagemann and Erdmann, 1994) in which a basal level of GgpS has subsequently been detected (Marin *et al*., 2002). Currently, GgpS and GgpP are known to become activated in direct correlation to ion strength, although some ions are more efficient activators than others (Hagemann and Erdmann, 1994; Schoor *et al*., 1999). Interestingly, the activation of GG formation by ions is reported for the freshwater organism *Synechocystis* and, as our data has indicated, probably for *D. shibae*, but not for the marine cyanobacterium *Synechococcus*, which obviously maintains a different regulation strategy (Engelbrecht *et al*., 1999; Empadinhas and da Costa, 2008a).

Growth phase dependence of GGA formation

As protein biosynthesis is strongly reduced at OD_{max}, the requirement of the usually indispensable glutamate as the nitrogen donor for amino acid biosynthesis and as a protein component is much smaller. Nevertheless, a sufficient turgor pressure must be maintained. Therefore, *D. shibae* is compelled to replace glutamate, a process performed by GGA and additional GG (Fig. 2). However, the balance between GG and GGA is directly dependent on the salinity of the medium. GGA accumulation is strongest under moderate salt conditions. In the exponential phase, however, glutamate is exclusively used as a compatible solute.

An increased concentration of GGA in the stationary phase has also been reported for *Halomonas elongata* (Cánovas *et al*., 1999). In *E. chrysanthemi* 3937, GGA becomes the most abundant osmolyte in stationary phase but its concentration continuously increases throughout growth (Goude *et al*., 2004). Such an accumulation of osmotically active substances during the transition into stationary growth has also been reported for trehalose in *E. coli* (Hengge-Aronis *et al*., 1991), which uses the disaccharide as an osmolyte (Giæver *et al*., 1988). However, in this case the function of trehalose as a storage compound has to be considered.

Further metabolic responses

An unexpected and interesting result of this study is the highly increased level of putrescine in *D.* shibae cells cultivated in low-salt medium [0.3% salts (Supporting Information Table S1)]. Polyamines such as putrescine, spermidine or spermine are known to be used to stabilize negatively charged macromolecules such as DNA, tRNA and ribosomes, partly as a replacement for the cation Mg^{2+} (Ohtaka and Uchida, 1963; Tabor and Tabor, 1976; Bachrach, 2005), and to influence interactions between proteins and nucleic acid (Record Jr *et al*., 1998). Under low osmolarity conditions, *E. coli* also exhibits an increased putrescine concentration (Munro and Bell, 1973). In *Pasteurella tularensis*, polyamine content has been reported to be reduced with increasing medium osmolarity (Mager, 1955). The balance between K^+-ions and putrescine^{2+}-ions in *E. coli*, has been shown to play a major role in the interaction of the *lac* repressor-*lac* operator (Capp *et al*., 1996). The small osmopressure at 0.3% salinity leads to a lower intracellular salt concentration and, possibly, putrescine partly takes the role of K^+ and Mg^{2+}. Thus, putrescine is accumulated to guarantee the functionality of macromolecules and their interactions. In addition, putrescine might also be used as a nitrogen-store. *D. shibae* has several enzymes with an aminotransferase class III domain and is therefore probably able to regenerate glutamate quickly by a transfer of an amino group to 2-oxoglutarate. In the case of a rapid change of salinity, glutamate would immediately be available for osmotic adjustment and as a nitrogen donor.

Under close-to-freshwater conditions, the high concentration of the fatty acid vaccenic acid in combination with 3-phosphoglycerol is notable (Supporting Information Table S1). Possibly, some adaptations of the cell membrane are necessary under these conditions and might be achieved by changes in the lipid or phospholipid composition. Further analyses of cell wall or lipid composition are needed but this is beyond the scope of our current study.

Additional metabolic regulations occur and are explained more easily. At 10 min after salt shock, charged molecules such as amino acids probably accumulate to counteract

the in-streaming ions and because of a temporarily strongly affected metabolism. Glycerol-3-phosphate is also upregulated (twofold) directly after salt shock (Supporting Information Table S1). This compound is needed for the synthesis of GG. Almost the same results have been observed at 90 min after the shock, but to a smaller extent.

As expected, nitrogen-limited cultures show a strong reduction of nitrogen-containing substances (Supporting Information Table S1). In general, the concentrations of most metabolites are decreased once OD_{max} is reached, indicating a disrupted metabolism.

Transcriptional response to salt shock

The transcriptional analysis of *D. shibae* after salt shock has revealed the immensely important role of the 153 kb chromid in osmoregulation: 42% of the chromid genes are upregulated at 10 min after salt shock and 23% of these genes are still upregulated at 90 min after the shock. The chromid carries numerous genes for transporters and channels, the genes for GG biosynthesis (Dshi_3830-3832) and a few regulated genes encoding hypothetical proteins. Furthermore, the histidine kinase located on the 153 kb chromid is regulated by a $\log_2$ fold-change of 5.6 at 10 min after the shock and of 2.7 at 90 min after the shock. In *Synechocystis* sp. PCC 6803, histidine kinases are known to regulate the expression of many salt-inducible genes (Marin *et al*., 2003). Our data show that the 153 kb chromid is essential for survival of *D. shibae* in the marine environment.

As expected, the genes for GG biosynthesis, namely GG-phosphate synthase and GG-3-phosphatase, are strongly upregulated immediately after salt shock, as are the genes encoding enzymes for the synthesis of glyceraldehyde-3-phosphate and glycerol-3-phosphate (Table 2). Glyceraldehyde-3-phosphate is the precursor for glycerol-3-phosphate, which is directly utilized for GG biosynthesis. As succinate was used as the sole carbon source in this study, an upregulation of gluconeogenesis would be expected in order to increase the formation of the GG precursor glucose. However, only the enzymes of the Entner-Doudoroff pathway were upregulated. This pathway consumes glucose, but produces glyceraldehyde-3-phosphate, which can be re-utilized for glucose or glycerol-3-phosphate formation. Because of the upregulation of the Entner–Doudoroff pathway, a production cycle for the educts of GG seems to be present. Transcriptional regulations has also been seen in the metabolome data, as glucose (fold-change 5.2) and glycerol-3-phosphate (fold-change 2.1) are both found to be increased 90 min after salt shock (Supporting Information Table S1).

Unlike GG, GGA formation seems to be independent of the applied salt stress, as only glucosyl-3-phosphoglycerate phosphatase is exclusively upregulated 90 min after salt shock. Hence, this regulation does not seem to be salt-dependent and is instead a secondary effect, a notion additionally supported by the low GGA concentration detected 90 min after the applied shock (Fig. 2).

Furthermore, at least five genes encoding known general stress proteins are upregulated throughout the experiment (Table 2). The two chaperons *clpB* (Dshi_0617) and the gene encoding the small heat shock (HSP20) protein (Dshi_2892) remain increased at 90 min after salt shock. As chaperons assist in the correct folding of proteins or mediate the disaggregation of stress-damaged proteins (Parsell *et al*., 1994), they are common stress responses, including osmotic stress. Further chaperons of *D. shibae* are not strongly regulated, but have generally high transcriptional levels (e.g. *groEL*, *dnaJ* and *dnaK*, Supporting Information Table S2).

The most strongly represented salt-specific regulations in *D. shibae* concern transporters and channels (Supporting Information Table S2), in particular 10 min after the salt shock. Notably, transcripts of mechanosensitive channels (Dshi_0743, Dshi_3905, Dshi_4182), proteins of the family solute/sodium symporter (Dshi_3906, Dshi_3914) and the transcript of the multi-anti-extrusion protein MatE (Dshi_3884) were strongly upregulated or reliably detected under shock conditions only (Table 2). Here, the cells react to the rapidly changed conditions by, for example, water efflux, changed turgor pressure and ion concentrations. The transcriptional response to an osmotic shock has been investigated in several organisms, such as *Bacillus subtilis*, *E. coli* and *Synechocystis* sp. PCC 6803, in which the crucial role of transporters has been shown (Roeßler and Müller, 2001; Marin *et al*., 2004; Fulda *et al*., 2006; Hahne *et al*., 2010)

An upregulation of t-RNAs has been observed, which implies the expected increase in protein biosynthesis during the adaptation process (Supporting Information Table S2).

D. shibae confronts saline environment – a model for Rhodobacteraceae?

As our study has shown, both GG and GGA play an essential role as compatible solutes in marine habitats, for *D. shibae* and for organisms of the same strategy-type. GG predominantly counteracts varying salt concentrations and the synthesis of GGA guarantees the maintenance of a moderate turgor pressure under nitrogen limitation, which is typical for the marine-conditioned lifestyle. With respect to marine conditions, adaptation strategies for osmoregulation must have evolved not only once in the family of *Rhodobacteraceae*. As not all analysed *Rhodobacteraceae* genomes possess the capability of forming GG and/or GGA, the production of other osmolytes such as carnitine, trehalose or ectoine is required to cope with

their saline environment. Hence, GG and GGA have been confirmed in their role as osmolytes and provide an additional strategy for confronting saline habitats to those previously known.

In summary, the strategy that *D. shibae* uses to cope with salinity and/or with nitrogen limitation is typical for particular groups within the *Rhodobacteraceae* but does not represent the archetype for the whole family. Nevertheless, the distinct limitation of GG and GGA formation with regard to the marine habitat has been shown. Most probably, the evolutionary genesis of GG and GGA was not from marine *Rhodobacteraceae*, as other phylogenetic groups such as the pseudomonades and some remotely related cyanobacteria possess also the genes examined in this study.

Conclusion

D. shibae DFL12^T is ideally prepared for dealing with its environment including salinity changes and nitrogen limitation. The organism uses glutamate, α-glucosylglycerol, α-glucosylglycerate and putrescine to confront osmotic stress. Putrescine is used under hypo-osmotic conditions to protect macromolecules and guarantee their functionality. Glutamate is the first-choice solute for osmoregulation and, under more challenging salt concentrations GG is used additionally. GGA, which has been found here for the first time within the α-proteobacteria, is used as a replacement of glutamate and is linked to special circumstances as indicated by the nitrogen-limitation experiment and the dependence on growth phase. Transcriptome analysis of *D. shibae* cells after salt shock has highlighted the essential role of the 153 kb chromid in the adaptation to changing salinity. Comparative genomics analysis has revealed that the formation of the compatible solutes GG and GGA is limited to the marine environment within the *Rhodobacteraceae* family.

Experimental procedures

Cultivation conditions and sampling time points

D. shibae DFL12^T was cultivated in defined salt water minimal medium (SWM, (Zech *et al.*, 2009) with 16.9 mM succinate as the sole carbon source and 4.7 mM ammonium as the nitrogen source under all conditions. Minimal salinity was 0.3% [expressed as NaCl (w/v) because of the necessary buffer substances and called basic salt mix 4.0 g $NaSO_4$, 0.2 g KH_2PO_4, 0.25 g NH_4Cl, 20.0 g NaCl, 0.19 g $NaHCO_3$, 3.0 g $MgCl_2$ $6H_2O$, 0.5 g KCl and 0.25 g $CaCl_2$ $2H_2O$ per litre medium]. Generally, salinity was regulated by adding a 166.75 g/L NaCl-solution to the basic salt mix up to the concentrations 2.3%, 3.5% and 5% (w/v). Cultivation was carried out at 30°C in baffled shaking flasks, at 150 r.p.m. under darkness. Pre-cultures were inoculated with previously salinity-adapted cells. The cells were harvested either at an OD_{600} of 1 or at the maximal OD_{600}. Salt shock was achieved by adding 2.7 g autoclaved NaCl to cultures initially cultured in medium containing 2.3% salts, thereby achieving an end concentration of 5%. Sampling occurred 10 and 90 min after salt shock; this was also the case in the reference culture cultivated constantly in medium with 2.3% salt. Even at 90 min after the first sampling time point, cells continued actively to grow. During nitrogen limitation, 1 mM ammonium was supplied. Salinity was 3.5% and sampling occurred at an OD_{600} of 1.

Sample preparation

Cells were harvested by centrifugation at 9000 *g* for 3 min at 4°C. Cells were washed twice with 0.9%, 2.3%, 3.5% or 5% NaCl-solution (w/v), according to the cultivation conditions of 0.3%, 2.3%, 3.5% or 5% salinity at various time points. Afterwards, cells were resuspended in 750 μl of an ethanolic adonitol solution (3 μg/ml) and lysed in an ultrasonic bath for 15 min at 70°C. After cooling on ice, 750 μl water was added and the mixture was homogenized. Non-polar substances and proteins were removed by chloroform (1 ml) extraction. In each case 750 and 250 μl of the polar phase were lyophilized and stored at −80°C until further preparation.

The two-step derivatization with methoxyamine hydrochlorid and MSTFA (N-methyl-N-trimethylsilyltriflouroacetamide) was performed automatically as described recently (Reimer *et al.*, 2014) using a Gerstel MPS 2 XL Twister (Gerstel, Mühlheim a. d. Ruhr, Germany).

GC-EI-MS

Gas chromatography (GC) was performed in a 7890 Agilent GC chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a VF-5ms + 5 m EZ-guard column (30 m, 0.25 mm inner diameter, 0.25 μm particle size, Agilent, Waldbronn, Germany). MS analysis was performed as described recently (Laass *et al.*, 2014) using a Leco Pegasus 4D GCxGC-TOF-MS (Leco Instrumente, Mönchengladbach, Germany) operated in GC-TOF mode and with a MPS 2 XL autosampler (Gerstel, Mühlheim an der Ruhr, Germany).

External calibrations of glutamate and β-glucosylglycerol performed. β-Glucosylglycerol was used for the quantification of α-glucosylglycerol and α-glucosylglycerate.

Data were processed with the metabolite detector software (Hiller *et al.*, 2009) and were normalized to cell mass and the internal standard adonitol.

GC-APCI-MS

Gas chromatography was performed in a 7890 Agilent GC chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a Zebron ZB-5MS + 5 m guardian column (Phenomenex®, Aschaffenburg, Germany) and a GC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The split ratio was adjusted according to the requirements of the samples (ratio 1:10–1:50). The transfer line was set to 300°C. MS analysis was performed with a Bruker micrOTOF-QII mass spectrometer (Bruker Daltonik, Bremen, Germany). Spectra were obtained in positive mode using a mass range of 100…1550 *m/z* and a spectra rate of 0.4 Hz. The settings of the MS instrument were: end plate offset, −500 V; capillary, −1000 V; corona, 3000 nA; nebulizer, 4 bar; dry gas, 4 l/min

and dry temperature, 180°C; funnel 1 RF, 200 Vpp; funnel 2 RF, 200 Vpp; in-source CID energy, 0 V; hexapole RF, 150 Vpp; quadrupole ion energy, 5 eV; collision energy, 7 eV; collision RF, 140 Vpp; transfer time, 80 μs and pre puls storage, 5 μs. For mass calibration an APCI/APPI Calibrant Solution (Fluka, München, Germany) was used. Data analysis was performed using DataAnalysis 4.0 (Bruker Daltoniks, Bremen, Germany).

Comparative genomics analyses

Protein sequence information of chromosomal and extrachromosomal elements of selected *Rhodobacterales* genomes (135, Supporting Information Table S3) were extracted from the nr-database of the NCBI (Sayers *et al*., 2011).

Protein sequences from *D. shibae* DFL12^T (glucosylglycerate synthesis: Dshi_1820, Dshi_1821, Dshi_1822; glucosylglycerol synthesis: Dshi_3830, Dshi_3831, Dshi_3832 [2.4.1.213]) were blasted against the built BLAST-database (Altschul *et al*., 1990; makeblastdb, ncbi-blast-2.2.31+).

Information about organism name, genelocus, startposition, endposition, direction, and whether chromosomal or not were assigned to sequence headers.

Concerned protein sequences from *D. shibae* DFL12^T (glucosylglycerate synthesis: Dshi_1820, Dshi_1821, Dshi_1822; glucosylglycerol synthesis: Dshi_3830, Dshi_3831, Dshi_3832 [2.4.1.213]) were blasted against the built BLAST-database (Altschul *et al*., 1990; blastp, ncbi-blast-2.2.31+).

Finally, the evolutionary versatility and dissemination of *Dinoroseobacter* elements in the *Rhodobacterales* clade were analyzed. Chosen cutoffs were an *E*-value smaller than 0.01 and no limitation of identity and alignment length to obtain an overview.

Protein domain architectures of genes involved in glucosylglycerol synthesis were examined in detail by a pretrained Hidden Markov Model (Haft *et al*., 2003) TIGR02398 and hmmscan domain results (Eddy, 1998).

Taxonomy and BLAST results were used to obtain an overview of gene distribution over the whole tree of life. BLAST and HMM results were mapped to habitat information of organisms (marine/non-marine) extracted from NCBI (Sayers *et al*., 2011). Salt concentrations equivalent to the marine environment were considered to be a comparable habitat. The identification, annotation and analysis of domain architectures of several genes in the organisms were performed with the help of SMART (Letunic *et al*., 2015).

16S-RNA sequences of 64 *Rhodobacterales* genomes with a predicted presence off GG and GGA synthesis genes were extracted from the NCBI (Sayers *et al*., 2011) or PATRIC (Wattam *et al*., 2013) for a phylogenetic analysis. For generation of the phylogenetic tree an analysis workflow of Mobyle Portal (Néron *et al*., 2009) was applied. MUSCLE 3.8.31 was used to align the RNA sequences in phylip format. A distance method tree (dna_bootstrap_distance_phylogeny, distance model:F84, bootstraps: 100, phylip 3.67: dnadist/neighbor) was concatenated (nw_cat GNU 7.4) with the maximum likelihood statistical approach (phyml 20130219.patch, nucleotide substitution model:HKY85, bootstraps:100) to obtain the resulting consensus tree (phylip 3.67: consense) visualized with the help of Archaeopteryx 0.9899 beta (Han and Zmasek, 2009). A seven-organisms group either taxonomically assigned to *Rhodobacteraceae* but rather clustering with *Rhizobiales* in 16S rRNA gene analyses or members of the sister *Stappia* group (Munoz *et al*., 2011; Pujalte *et al*., 2014) were used as outgroup for rooting.

Transcriptome analysis using RNA-Seq

The quality and integrity of the total RNA was controlled on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The RNA sequencing library was generated from 1000 ng total RNA after rRNA depletion by RiboZero followed by ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre) according to the manufacturer's protocols. The libraries were sequenced on Illumina HiSeq2500 using TruSeq SBS Kit v3-HS (50 cycles, single ended run) with an average of 3×10^7 reads per RNA sample. Reads were aligned to the reference genome (Dinoroseobacter shibae DFL 12 uid58707) using the open source software Rockhopper.

Phenotype MicroArray analysis (Nitrogen Sources)d

To identify usable nitrogen sources, Phenotype MicroArray analyses were performed following the manufacturers' instructions (Biolog Inc., USA) with modifications. The inoculation and incubation solutions IF-0a GN/GP were adapted to the artificial sea water medium as described (Rex *et al*., 2013) by adding a 10-fold concentrated SWM including vitamins and trace elements. Succinate was supplied as the carbon source (final concentration of components equal to SWM), while nitrogen was excluded. Data analysis was done as described recently by (Rex *et al*., 2013).

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The authors declare that they have no conflicts of interest.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1: Metabolites in *D. shibae* DFL12^T grown under different salinities (0.3–5%), after a salt shock (2.3 to 5% salinity) and nitrogen limited (salinity 3.5%). Long-term adapted cells were sampled in the exponential growth phase and at a maximal optical density (OD_{max}). Nitrogen limited culture was exclusively sampled during active growth. These data were compared to the according growth situation in a culture grown in medium with a salinity of 3.5%. Salt shocked cells were harvested 10 and 90 min after the shock and were compared to untreated reference culture (continuous 2.3% salinity) at the same time points.

Table S2: Transcriptome data of *D. shibae* DFL12^T salt shocked. Initial medium salinity was 2.3% and final salinity was 5%. The shock was performed in the mid exponential growth phase. Data were compared to an untreated reference culture.

Table S3: List of organisms included in comparative genomics analysis, comprising of 28 not marine, 101 marine and four further members of the family *Rhodobacteraceae*.